followed by ovum implantation into a pseudopregnant female, etc.). The Cas9 protein is under the control of a promoter that expresses at least in embryonic stem cells, and may be additionally under temporal or tissue-specific control (e.g., drug inducible, controlled by a Cre/Lox based promoter system, etc.). Once a line of transgenic Cas9 expressing mice is generated, embryonic stem cells are isolated and cultured and in some cases ES cells are frozen for future use. Because the isolated ES cells express Cas9 (and in some cases the expression is under temporal control (e.g., drug inducible), new knock-out or knock-in cells (and therefore mice) are rapidly generated at any desired locus in the genome by introducing an appropriately designed DNA-targeting RNA that targets the Cas9 to a particular locus of choice. Such a system, and many variations thereof, is used to generate new genetically modified organisms at any locus of choice. When modified Cas9 is used to modulate transcription and/or modify DNA and/or modify polypeptides associated with DNA, the ES cells themselves (or any differentiated cells derived from the ES cells (e.g., an entire mouse, a differentiated cell line, etc.) are used to study to properties of any gene of choice (or any expression product of choice, or any genomic locus of choice) simply by introducing an appropriate DNA-targeting RNA into a desired Cas9 expressing cell.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

SEQUENCE LISTING

The patent contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US10227611B2). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

The invention claimed is:

1. A method of modifying a target DNA molecule in a cell, the method comprising contacting a target DNA molecule inside of a cell with:

(a) a Cas9 protein; and

(b) a single molecule DNA-targeting RNA comprising, in 5' to 3' order:

(i) a targeter-RNA that hybridizes with a target sequence of the target DNA molecule,

(ii) a nucleotide linker; and

(iii) an activator-RNA that hybridizes with the targeter-RNA to form a double-stranded RNA duplex,

wherein (a) forms a complex with (b) and the target DNA molecule is modified.

2. The method of claim **1**, wherein the target DNA molecule is modified by being cleaved.

3. The method of claim **1**, wherein the method comprises introducing into the cell:

(1) the single molecule DNA-targeting RNA, or a DNA molecule that encodes the single molecule DNA-targeting RNA; and

(2) the Cas9 protein, an RNA molecule that encodes the Cas9 protein, or a DNA molecule that encodes the Cas9 protein.

4. The method of claim **3**, wherein the DNA molecule that encodes the single molecule DNA-targeting RNA and/or the DNA molecule that encodes the Cas9 protein is a recombinant expression vector.

5. The method of claim **3**, wherein the method comprises introducing into the cell a DNA molecule that encodes both the single molecule DNA-targeting RNA and the Cas9 protein.

6. The method of claim **3**, wherein the RNA molecule or the DNA molecule of (2) comprises a nucleotide sequence that is modified relative to a corresponding wild-type nucleotide sequence encoding a Cas9 protein, wherein the modification replaces one or more codons in the wild-type nucleotide sequence with one or more different codons encoding the same amino acid.

7. The method of claim **3**, wherein the method comprises introducing a donor polynucleotide into the cell.

8. The method of claim **3**, wherein the method comprises introducing two or more single molecule DNA-targeting RNAs into the cell, wherein the two or more single molecule DNA-targeting RNAs are complementary to different target sequences within the same or different target DNA molecules.

9. The method of claim **8**, wherein the two or more single molecule DNA-targeting RNAs are introduced into the cell at the same time.

10. The method of claim **1**, wherein said contacting comprises inducing expression of (a) and/or (b).

11. The method of claim **1**, wherein the Cas9 protein comprises one or more mutations in a RuvC domain and/or a HNH domain.

12. The method of claim **1**, wherein:

the targeter-RNA comprises the 12 nucleotide (nt) crRNA sequence GUUUUAGAGCUA (SEQ ID NO: 679),

the nucleotide linker comprises the 4 nt sequence 5'-GAAA-3', and

the activator-RNA comprises the 67 nt tracrRNA sequence 5'-UAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUC GGUGCUUUUUUU-3' (SEQ ID NO: 432).

13. The method of claim **1**, wherein (i) comprises the crRNA sequence GUUUUAGAGCUA (SEQ ID NO: 679) and (iii) comprises the tracrRNA sequence 5'-UAGCAAGUUAAAAUAAGGCUAGUCCG-3' (SEQ ID NO: 397).

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